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The hypothesis being tested in this project is that a greater proportion of patients who develop radiation-induced RTOG/EORTC grade 4 subcutaneous late tissue morbidity possess a mutated ATM gene compared with patients who do not suffer these complications. An additional objective is to determine the functional impact upon the protein encoded both in the ATM gene for each mutation identified and subsequent cellular radiosensitivity.

The specific aims of this project are to (1) screen 50 breast cancer patients for a ATM mutations who developed radiation-induced grade 4 late subcutaneous tissue morbidity as defined by the RTOG/EORTC scoring scheme, (2) establish control group and screen 100 patients without evidence of this late radiation reaction, and (3) perform functional studies using cells from patients identified as ATM carriers to determine to what extent each ATM mutation identified affect radiosensitivity and normal activity of the protein produced by the ATM gene.

The main accomplishment during the first year of this study was to establish and optimize all of the assays and technique that will be used in this project.

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## INTRODUCTION

The majority of female breast cancer patients treated with breast conservation protocols consisting of limited surgery followed by adjuvant radiation therapy to the breast and surgical bed can develop tissue changes within the irradiated volume. These changes are both expected and temporary, and in most instances will resolve with conservative medical management. In contrast, there is a small subset of patients who manifest persistent or late subcutaneous tissue changes that can result in poor cosmesis and often painful sequelae. In some cases there are plausible explanations for such reactions that may include large breast size, excessive radiation dose-fractionation schedules, use of concurrent chemotherapy, and medical comorbidities such as collagen vascular diseases and diabetes. However, there exists an important subset of patients with no clear explanation for excessive post-treatment morbidity and the potential for a genetic basis must be considered. The purpose of this study is to investigate whether the *ATM* gene plays a role in enhanced radiation sensitivity in this population. This gene was selected because the protein it encodes plays a critical role in the response of cells to radiation and the repair of radiation-induced damage. Furthermore, cells possessing a mutated copy of this gene are more radiosensitive than cells from individuals with a normal genotype. In addition, the results of a pilot study screening breast cancer patients are supportive of the hypothesis that patients who are carriers of an *ATM* mutation are more likely to develop late radiation-induced subcutaneous tissue complications.

The principal goal of this project is to determine whether women who inherit a mutated copy of the *ATM* gene are more prone to the development of late radiation-induced morbidity. This will be accomplished through comprehensive screening of the *ATM* gene for germline mutations. If a correlation is found between radiosensitivity and *ATM* mutation status, this would indicate that possession of an altered *ATM* gene results in susceptibility to subcutaneous tissue complications for breast cancer radiotherapy patients. In addition, a determination will be made as to the pathogenic consequences of each *ATM* mutation through the use of functional studies that will examine the *ATM* protein in cells from patients who are carriers of a mutation in this gene. This project is innovative as it represents the first study to use the powerful DHPLC mutation screening technique to investigate the association between *ATM* heterozygosity and radiation-induced morbidity in the female breast cancer population. It is also the first study to examine whether there is a correlation between the presence of specific *ATM* mutations, development of radiation-induced complications, and impairment of *ATM* protein function based upon cellular and molecular analyses.

Confirmation of this hypothesis will have important and direct implications upon patient care. It may suggest that all newly diagnosed female breast cancer patients considering breast conservation management should be tested for *ATM* heterozygosity using the relatively rapid and efficient mutation screening approach outlined in this proposal. Those women found to harbor an *ATM* mutation may not be ideal candidates for standard breast conservation protocols and could possibly be better served by alternate treatment approaches such as modified radical mastectomy and breast reconstruction. Alternatively, these women may be ideal candidates for a dose reduction trial. A reduced total dose to the breast may result in equivalent local control rates as germline *ATM* gene alterations should be present in both tumor and normal cells and cause enhanced radiation sensitivity for both cell types. However, this remains to be tested. In either case, *ATM* mutation detection may help to prevent many women from experiencing the poor cosmetic and potentially painful side effects that can result from conventional breast radiotherapy in *ATM* carriers.

## Body

Progress to obtain DNA samples from subjects was limited for the first year as it was not possible to accrue any subjects into our study since we still do not have final approval from the HSRRB (Human Subjects Review Board) of the DOD for the human subjects protocol and informed consent forms. Although every effort was made to expedite this process during the past year, tentative approval was only received on April 30, 2003, that is contingent upon approval from both the Mount Sinai and NYU IRBs. The process to obtain local IRB approval was only then initiated as I was instructed not to pursue approval from the local IRBs until this material was approved by the HSRRB. At this time, final IRB approval for this project has been given by the Mount Sinai and tentative approval by the NYU IRB with several minor modifications requested.

However, a significant amount of work was accomplished in the past year to establish and optimize the techniques being used in this project for both the genetic screening and functional studies. It should be noted that this work was essentially divided between this project and grant DAMD17-02-1-0502.

In terms of the genetic analysis, a high throughput modification for our WAVE DHPLC system, the technique that will be used for genetic analysis of the DNA samples, was instituted during the past year. This upgrade of our system permits a reduction from 10, to approximately 3 minutes, to accomplish the analysis of each sample, thereby substantially increasing the speed of sample screening. In addition, this modification to the WAVE system appears to also enhance the sensitivity of the DHPLC technique. However, because of this modification, it was necessary to re-optimize all of the temperature and buffer conditions under which the DHPLC is performed for each exon. This work is now complete and we have established the optimal conditions for detection of genetic variants using our high throughput upgraded WAVE system. In addition, we began using the enzyme Optimase, primarily because it has been designed specifically for production of PCR products to be screened using the WAVE system. However, this necessitated defining the optimal PCR temperature conditions for each of the 62 exons using this enzyme.

Work was also initiated to perform the functional studies using wild type and *ATM* mutant lymphoblastoid cell lines. The first part of this work has been the immunodetection of total p53 and p53 which has been phosphorylated. Since it is known that ATM phosphorylates p53 at ser-15, and given that there are commercially available antibodies that specifically recognize p53 protein that has been phosphorylated at ser-15, it is possible to determine the kinase activity of ATM in various cell types indirectly by determining the percentage of total p53 in a cell that has been phosphorylated by ATM at ser-15.

Briefly, we sediment the cells and then resuspend the cells in lysis buffer (50mM Tris/HCl, 5mM Na<sub>2</sub>EDTA, 150 mM NaCl, 0.5% NP40, 1mM DTT, 1mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM sodium orthovanadate, 1mM sodium fluoride) for 20 minutes on ice. The protein concentration is determined using a modified Bradford assay kit (RC-DC, BioRad). 1mg of cellular protein lysate is incubated with 1 µg of monoclonal anti-p53 (5 µl of a 200 µg/ml stock of 1C12 Mouse Monoclonal #2524, Cell Signaling technology) for 1 hour at 4°C with agitation. Protein-A conjugated to Sepharose beads (Sigma) is added to the mixture and incubated for 1 hr. at 4°C with agitation. The immunocomplex is precipitated by centrifugation, washed with 5 volumes of lysis buffer five times, and boiled in 20 µl Laemmli SDS-PAGE loading buffer (62.5 mM Tris/HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol) (BioRad). The beads are pelleted again using centrifugation.

The supernatant (containing the protein) is loaded onto a 7.5% precast gel (BioRad) and electrophoresed until the bromophenol blue dye front reaches the end of the gel. The proteins on the gel are transferred to a PVDF membrane (Immuno-Blot, BioRad) in Towbin buffer (25mM Tris/HCl, 192 mM glycine, 20% methanol, BioRad) for 2 hr at 4°C. The membrane is allowed to dry and washed in 25 ml phosphate buffered saline with 0.1% Tween-20 (PBST) twice for 5 min at room temperature (RT). The membrane is blocked with 25 ml PBST buffer with added protein blocker for 1 hr at RT and washed twice with 25 ml PBST for 5 min at RT. To the blocked membrane is added 10 ml of antibody dilution buffer (ADB) containing polyclonal rabbit Phospho-p53 (Ser15) antibody (Cell Signaling technology #9284, diluted 1:2000 [5 µl to 10 ml ADB]) and incubated for 1 hr at 4°C with rocking. The blot is washed twice in 25 ml PBST at RT and incubated for 1 hr at 4°C with rocking in secondary antibody (goat-anti-rabbit conjugated with horseradish peroxidase [GAR-HRP]) diluted to 1:5000 in ADB (2 µl antibody added to 10 ml ADB).

The blot is washed twice in 25 ml PBST at RT. The membrane is incubated in diluted BioRad Amplification Reagent (BAR) for 10 min at RT with rocking, washed four times in PBST with 20% DMSO 5 min at RT, and twice in PBST 5 min at RT. The membrane is incubated in diluted streptavidin-HRP for 30 min, followed by two washes in PBST 5 min RT. Using the Opti-4CN colorimetric detection from the BioRad kit, 15 ml of the colorimetric solution is prepared and the blot incubated in this solution for about 30 min or until the desired level of sensitivity is attained. The blot is washed in ddH<sub>2</sub>O for 15 min. The colorimetrically labeled blot is scanned using a table scanner and each band in the scan quantitated using ImageJ (public domain software downloaded from <http://rsb.info.nih.gov/ij/>). The blot is stripped using 40% acetonitrile, following which it is reprobed using the above conditions, but substituting the polyclonal rabbit Phospho-p53 (Ser15) antibody with polyclonal p53 Antibody (#9282 Cell Signaling Technology). Using numerical values representing the relative amount of each form (whole total p53 protein or Ser-15 phosphorylated p53), a ratio of the two is calculated, determining the percentage of the total p53 protein present in the cell which is phosphorylated at ser-15.

Work was also performed during the past year to establish the assay for immunodetection of ATM protein to determine the level of ATM present in various cell types. To accomplish this, the wild type and ATM mutant cells are precipitated by centrifugation and lysed directly in Laemmli SDS-PAGE sample buffer. The protein level is then quantitated using a Bradford protein assay. For each sample, the Western blot procedure described above is employed, though the antibodies used are as follows: for the first probe, rabbit polyclonal anti-ATM antibody (H-280, sc-15392, Santa Cruz Biotechnology, diluted 1:500 in ADB), and for the second probe, rabbit polyclonal anti-β-Actin (H-196, sc-7210, Santa Cruz Biotechnology, diluted 1:500 in ADB). As β-Actin levels remain constant in cells under a variety of conditions, the quantitation values obtained for the β-Actin probe are used to normalize the ATM values, so an accurate comparison of relative levels of the ATM protein can be made for each cell type.

## **Key Research Accomplishments**

- Establishment of a high through-put enhancement to the WAVE DHPLC system which will be used for this project.
- Optimization of DHPLC buffer and temperature conditions for mutation/polymorphism detection using the high throughput system.
- Optimization of the PCR temperature conditions using Optimase.
- Establishment of the assay for quantitation of p53 phosphorylation on ser-15 by ATM.
- Establishment of the assay for ATM protein level quantitation.

## **Reportable Outcomes**

None

## **Conclusions**

Accrual of patients into this study was limited during the first year of the project due to the lengthy process to obtain approval from the HSRRB for the human subjects protocol and consent forms. However, substantial progress was made to establish and optimize the assays and techniques to be used in this project. We are therefore now in a good position to rapidly accomplish all of the tasks outlined for this project.

## **References**

None

## **Appendices**

None